THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: January 22,19

1. Principal Investigator (give title and degrees):

H. Hugh Fudenberg, M.D.
Professor of Medicine, UC San Francisco
Professor of Bacteriology and Immunology, UC, Berkeley

D. Michaeli, Ph.D. (co-Investigator)
Assistant Professor of Biochemistry, UC, San Francisco

2. Institution & address:

University of California
Section of Hematology and Immunology
475 HSW
Third and Parnassus Streets
San Francisco, California 94122

3. Department(s) where research will be done or collaboration provided:

Section of Hematology and Immunology - Department of Medicine

4. Short title of study:

COLLAGEN ANTIBODIES IN RELATION TO THE ETIOLOGY OF EMPHYSEMA

5. Proposed starting date: March 31, 1973 (renewal date)

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

1. To ascertain the incidence of antibodies to collagen in the sera of smokers and non-smokers with chronic obstructive pulmonary disease (COPD) in control populations matched for age, sex, ethnic origin and smoking history. 2. To investigate the incidence of such antibodies in asymptomatic first-degree relatives of patients with COPD to see if such antibodies indicate genetic predisposition of this disease. 3. To see whether such antibodies bind to lung tissues of affected individuals, thus indicating that they may be a cause rather than a consequence of the disease. 4. To study certain lysosomal enzymes in pulmonary macrophages of patients with COPD and controls (other pulmonary diseases), especially cathepsin D&E, which cleave collagen. 5. To ascertain whether, and if so which, components of cigarette smoke bind to collagen to produce an immunogenic carrier-hapten complex. 6. To determine the three dimensional structure of normal human lung collagen and compare it with collagen from emphysematous lungs.

8. Brief statement of working hypothesis:

We have found anticollagen antibodies in a high percentage of patients with chronic obstructive pulmonary disease. Whether these are the cause of the disease, that is bicollagen breakdown, whether they arise due to damaged collagen or whether they indicate genetic predisposition to the disease is uncertain. Family studies of the three dimensional normal collagen and of collagen in which antibody is fixed in vivo should help answer this question.

Since macrophages are involved in the first stages of immunologic reactions and since macrophages contain enzymes which probably degrade lung collagen, studies of macrophages of smokers and nonsmokers with or without emphysema, including various enzyme levels, and the correlation with the presence or absence of antibodies will shed additional light on the role of macrophages in the etiology of emphysema.

9. Details of experimental design and procedures (append extra pages as necessary)

Please see attached pages.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

2400 square feet - 495 HSW UC Medical Center
1000 square feet - 839 HSE UC Medical Center

Approximately 2,400 square feet of laboratory space is available with cold room facilities, fraction collectors, starch gel, acrylamide gel and immunoelectrophoresis equipment. A Spinco Model 120 C automatic amino-acid analyzer equipped with automatic sample injector and integrator, a Model E analytical ulracentrifuge and equipment for paper chromatography are also available in our laboratory, and we have a separate room for high voltage paper electrophoresis (with proper precautions to avoid laboratory accidents). A Spinco Model 890 protein sequencer is also in use.

11. Additional facilities required:

none. Salaries for faculty and professional research personnel are based on the current July 1972 salary appropriations. A yearly merit increase of 5% and a 5% range adjustment is also reflected in the salaries of nonacademic personnel.

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12. Biographical sketches of investigator(s) and other professional personnel (append): Dr. Pinto da Silva and Dr. Melvin Schanfield are no longer listed on this grant. Dr. John Belton and Dr. J. L. Caldwell have been added. Biographical sketches of both doctors are attached.

13. Publications (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See references attached to progress report. Copies of "The Freeze-Etch Structure of Lung and Tendon Collagen in Young Rabbits [illegible]

9A. METHODS;

1. Immunochemical Studies on Collagen:

Polypeptide chains and β components of collagen will be separated by chromatography on CM-cellulose as described by Piez et al. (1). The separated chains will be treated with cyanogen bromide* and the resulting peptides will be separated on phosphocellulose (2). The advantage of this procedure over proteolytic digestion of the polypeptide chains is that, in addition to isolation and identification of the determinants, it will be possible to determine their location in the chain since the order of the CNBr fragments of several collagens has recently been determined (3). The following procedures will be used for assay of antigenic activity of α chains and β components as well as of CNBr fragments of chains:

(a) Precipitin reaction, as described by Michaeli et al. (4), will be used for assays of α and β chains.

(b) Hemagglutination and hemagglutination-inhibition, using the chromic chloride system of Gold and Fudenberg (5) will be used for assay of antigenic activity of the CNBr peptides.

(c) Collagen will be labeled with H^3-glycine by incorporation of the radioactive amino acid in the diet and the labeled chains of collagen will be treated with CNBr as described above. The small peptides, (M. V. 1500-5000), derived from the amino terminal end of the α chains will be assayed by equilibrium dialysis--a technique that will provide quantitative information both on the amount of antibodies directed to a specific peptide and on their binding constants with the peptides. Large, non-dialyzable peptides, will be assayed by the ammonium sulphate precipitation technique (6) yielding essentially the same type of information with regard to amounts of antibodies and estimates of binding constants.

Free collagen and collagen breakdown produced in the serum will be assayed by inhibition of precipitation of radiolabeled antigen

* Hereafter, cyanogen bromide will be abbreviated as CNBr.

using the method of Levin and Fudenberg (7). The amino acid sequence of peptides possessing antigenic activity will be determined by the Edman setpwise cleavage from the NH_2-terminal end of the peptide as modified by Crestfield et al. (8). Degradations of the carboxy-terminal end will be carried out with carboxypeptidase A and B. Intermediate peptides will be assayed for immunological activity. Immunologically active peptides will be synthesized by the solid phase peptide synthesis (9). The synthetic peptides will be assayed as described above. Also, analogues of the peptides will be synthesized and assayed for immunological activity in order to ascertain the relationship between structure and antigenic activity. Collagen may be converted from a relatively poor antigenic entity into a potent antigen by attachment of foreign chemical groups on it. The presence of anti-collagen activity in sera of some emphysema patients (Fudenberg, and Michaeli, in preparation) suggests that such a mechanism may be operative in this condition. (Identification of the chemical entities in the gaseous phase of cigarette smoke that have the capacity to bind to lung collagen may prove essential in the understanding of the etiology of this disease.)

2. Serologic Studies:

Sera will be obtained from 300 patients with COPD, as defined by pulmonary function tests (Appendix 1), and from 300 normal control patients matched for age, sex, ethnic origin, and smoking history. Sera will also be obtained from 300 patients with other forms of pulmonary disease though obtaining matching sera by the parameter outlined above may not be feasible. Sera of first degree relatives on whom pulmonary function studies have been done will also be tested.

These sera will be assayed via human collagen and altered collagen, and altered peptides by the chromic chloride hemagglutination method of Gold and Fudenberg (5). The γ-globulins will be isolated from selected sero-positive (and control sero-negative) sera and Fab (antigen-binding) and Fc fragments produced by papain digestion to insure that the antigen-binding is indeed via the Fab fragment (the portion of the molecule which binds to antigen)

rather than being nonspecific. The whole sera, IgG, and Fab fragments will also be reacted with a synthetic peptide of charg closely similar to collagen, namely poly tyrosyl-glutamyl-argunyl lysine (T, G, A, L) to insure that the reaction occurring is indeed immunologic and not merely due to protein - protei interaction on a "charge" basis.

3. Cellular Immunity Studies:

In the past few years, it has been evident that response of immunologically competent cells (i.e., immune response at the cellular level) is a better parometer of immunologic reactivity to various antigen than is serum antibodies. We will measure cellular immune response to collagen, to modified collagen, and to collagen peptides (in addition to serum antibody) by the following parometers of cellular immunity:

A) Lymphocyte response to antigen, as measured by incorporation of labeled processor, (Usually H^3 - thymidine into DNA) using a synchronized cell in with incorporation of same (10).

B) Hemolysis in gel of red cells coated with antigen by single lymphocytes or plasma cells in the presence of complement, the so-called "Jerne plaque technique" (11).

C) Inhibition of migration in glass by normal macrophages in the presence of both antigen and lymphocyte from sensitized donor - e.g., migration inhibitory factor (MIF) (12).

(The MIF studies will be performed by Dr. Levin, the lymphocyte studies by Dr. Fudenberg, and the Jerne plaque studies by Dr. Henry.)

4. Electron Microscopic Studies:

After reduction, pepsin fragments of IgG anti-collagen and anti-ferritin will be prepared, antibodies to collagen will be hybridized (13a) with antibodies to ferritin and the hybrid antibody used in attempts to demonstrate collagen antibodies fixing to lung tissue by immuno electron microscopy (13b).

4.(b) We also propose to study human lung collagen, using the freeze fracture technique, from normal and emphysemous lung in the same way we have studied rat tendon and lung collagen to establish the normal and three dimensional structure in emphysema and the deviations from it, if any.

5. Pulmonary Macrophage Studies:

Pulmonary macrophage obtained by bronchial aspiration from, patients with COPD and from controls with other pulmonary disease (donors will be obtained during bronchial aspiration and lavage; lysosomal enzyme levels determined by classic chromic assays (14). Special attention will be paid to cathepsin D & E, since evidence exist that these digest collagen (15,16); other lysosomal enzymes (e.g., RNAase, acid phosphotase, etc.) will be measured as controls, especially lysozyme, has anti bacterial properties. (Dr. Hanes, who has had extensive experience with lysosomal enzyme purification and preparation in quantitive measurements will perform these measurements.) Levels of lysosomal enzymes which cleave mucopolysaccharides, (1) Hyaluronidase, (2) β-Glucoronidase, (3) Sialidase, (4) β-Galactosidase, α-Galactosidase, and (5) μ-Acetyl - β-Glucosaminadase, (17) will be measured in view of the obvious relevance to the intracellular cement substance in the lung.

B. SIGNIFICANCE:

1. Antibodies to Collagen:

With regard to the anti-collagen antibodies as possible laboratory markers of genetic susceptibility to development of COPD analogies exist with other antibodies; antibodies to collagen may be consequence, cause, or merely laboratory markers of genetic susceptibility to development of COPD.

Release of collagen into the induction of antibodies to denatured lung collagen may be incidental to the destruction of the lung parenchyma. This possibility is not very likely in view of the low immunogenicity of collagen and the massive doses of collagen required for induction of anti-collagen production (18).

Formaldehyde, which is present in the gaseous phase of cigarette smoke (19), may react with collagen and introduce an increased amount of intermolecular cross-linking. This would have the double-effect of lowered permiability of alveolar walls and increased antigenicity of the collagen. Other compounds in the gaseous phase of cigarette smoke (acetaldehyde, ammonia, acrolein) may react with collagen. Such an interaction may convert collagen from a relatively poor antigenic entity into a potent antigen (20).

Insofar as being laboratory markers of genetic predisposition to COPD, the analogy of anti-collagen antibodies to other antibodies is pertinent. For example, in asymptomatic relatives of patients with rheumatoid arthritis, there is a high incidence

of antibodies to γ-globulin (rheumatoid factors) of the same type that is present in the vast majority (about 85%) of patients with rheumatoid arthritis. Thus, it appears that the rheumatoid factor is a genetically determined serologic marker capable of detecting that proportion of the population which is genetically predisposed to develop rheumatoid arthritis upon exposure to certain bacterial, viral or other environmental agents (21).

If anti-collagen antibodies are found to be present in high frequency in asymptomatic relatives of patients with COPD, this would provide evidence of a genetic mechanism analogous to the rheumatoid factors. If so, asymptomatic individuals with such antibodies should be discouraged from smoking.

If the antibodies are restricted to the patients and not found in asymptomatic relatives, the question as to whether they are cause or effect then becomes relevant. Demonstration of fixation of such antibodies to lung tissue of patients with COPD but not to similar tissue of patients with other types of lung disease would provide evidence for a pathogenetic role for such antibodies.

Thus, an extensive survey of emphysema patients for anti-collagen antibodies and also titers and of controls matched according to sex, age and smoking habits appears warranted. If a high incidence of such antibodies is indeed present in COPD, we will isolate and identify the antigenic determinants of lung collagen as outlined in "Methods". Furthermore, we will ascertain whether or not lung collagen from emphysema patients is bound by haptenic material. For elucidating this problem, a variety of approaches will be utilized, e.g., measurements of free ε amino groups in collagen from normal and emphysematous lungs, assay of bound formaldehyde in lung collagen, assay of acetyl groups in lung collagen, etc., as outlined in "Methods".

2. Enzyme Studies:

Since parenchymal tissues and alveolar wall of the lungs contain large amounts of collagen, and, since cathepsin D & E may cleave collagen (22), it would be of considerable interest to see if cathepsin levels are increased in pulmonary macrophages of subjects with COPD. Further, cathepsin degrades other tissue protein which may be involved in the integrity of the lung. It has been shown that collagenase secreted extracellularly breaks up long collagen fibers which are then taken into phagosomes for digestion (22). Further, collagen fibers have been identified within lysosomes and digestion of collagen confirmed (23). The mucopolysaccharides are the intracellular cement of the lung

tissue. Increase in certain lysosomal enzymes in macrophages of COPD would theoretically aid in destructive tissue and development of emphysema.

Of the known lysosomal enzymes, the following cleave mucopolysaccarides (24,25):

1) Hyaluronidase

2) β-Glucoronidase

3) Sialidase

4) β-Galactosidase, α-Galactosidase

5) μ-Acetyl - β-Glucosaminadase

Hyaluronidase is known to be present in macrophages (25), as is β-Glucoronidase. The others have not been specifically looked for in macrophages but are probably present.

REFERENCES

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5. Gold, E. R., and Fudenberg, H. H., J. Immun., 99: 859, 1967.

6. Minden, P., and Farr, R. S., in Handbook of Experimental Immunology (Edited by D. M. Weir). F. A. Davis, Philadelphia, Pa., p. 463, 1967.

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17. Spiro, R. G., New Eng. J. Med. (Review), 281: 1043, 1969.

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25. Neufeld, E. F., and Frantantoni, J. C., Science (Review), 169: 141, 1970.

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BIOGRAPHICAL SKETCH
of
Dr. John C. Belton, M.S., Ph.D.

Personal:

Name -- JOHN CLAIR BELTON

Place and Date of Birth -

General Health -

Marital Status

Children -

REDACTED

Educational Data:

Undergraduate training in biological science was obtained at Lewis and Clark College in Portland, Oregon.

I spent the year at Colorado State University School of Veterinary Medicine.

Work was completed for a Master of Science degree at Oregon State University with a major in zoology.

Graduate studies leading to a Doctor of Philosophy degree were continued concurrently with a teaching assistantship. This degree was completed in June of 1966 and emphasized cell morphology and physiology.

REDACTED

Professional Employment:

1956-1957 -- Undergraduate laboratory assistant: Drosophila genetics, Bacteriology, etc.

1959-1964 -- Graduate teaching assistant for courses in general zoology, histology, cell biology, experimental embryology, genetics microtechnique.

1962-1964 -- I produced and organized teaching materials used in general zoology and histology laboratories. This job required a broad understanding of biological photography and microtechnique as well as teaching experience.

1964-1965 -- Instructor at Oregon State University, involved with the coordination of all general zoology laboratories for more than six hundred students. Duties also included specimen preparation, equipment maintenance and production of teaching materials.

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Professional Employment: (Continued)

1966-1969 -- Assistant Professor, California State College, Hayward, I have organized courses in comparative vertebrate histology, cell physiology and microtechnique. I have taught a graduate course on cell differentiation and morphogenesis

1969-1970-- Research Associate (Department of Botany, University of California, Berkeley). I held a research grant from the California Department of Public Health for the study of changes in lung fine structure resulting from exposure to nitrogen dioxide. This research was done in collaboration with Dr. Daniel Branton and provided one year training in the freeze-etch technique.

1970- Present Associate Professor, California State College. Hayward. I have been active in directing graduate student research.

1973 - Assistant Research Immunol.,Lecturer, UC,San Francisco Pending

Research Interests and Publications:

Belton, John C. and Alfred Owczarzak (1968). Cellular changes associated with the pre-ovulatory deposition and storage of hepatic lipids in the frog Ascaphus truei. Herpetologica 24: 113-127.

Belton, John C., D. Branton, H. V. Thomas and P. K. Mueller. (1971) Freeze-etch observation of Rat Lung. Anatomical Record.

Belton, John C. and C. M. Belton. (1971) Freeze-etch and Cytochemical Studies of the Integument of Larval Acanthatrium oregonense. Journal of Parasitology. 57: 252-260

Belton, John C. and Carol M. Belton (1970). Morphogenesis of Larval Integument of Acanthatrium oregonense. Second International Congress of Parasitologists, Washington, D. C.

Belton, J. C., McKally, and Fudenberg, H. H. (Submitted) Freeze-etch structure of lung collagen in young rats. J. Ultrastr. Res.

Belton, J. C., Tueller, E. E., McLaughlin, R. F. and Fudenberg, H. H. (submitted) Morphological changes in collagen associated with early human emphysema. Arch. of Path.

Professional Societies and honors:

REDACTED

Other:

Transcripts and other information will be furnished upon request.

DR. JOHN C. BELTON
Department of Biology
California State College
Hayward, California 94542

January, 1973

CURRICULUM VITAE

Joseph Lee Caldwell, M.D.

Birthdate: REDACTED

Education:

B.S.;M.D.	Arkansas State University, Jonesboro, Arkansas, University of Arkansas School of Medicine, Little Rock,	REDACTED

Training:

Intern	St. John's Hospital, Tulsa, Oklahoma, 1966-1967	
Resident	University of Iowa Hospital, Iowa City, Iowa, 1969-1971	
Research Fellow	University of Iowa and Veterans Administration Hospitals, 1971	

Positions:

United States Air Force Medical Officer Arizona and Viet Nam	1967-1969
University of Illinois, Dept. of Biochemistry Fellow	1972-1973
University of California, San Francisco	To be Appointed

14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
R. McLaughlin, Jr., M.D. Res. Assoc.	20%	
John Belton, Ph.D., Asst. Res. Immunol. I	50%	
D. Hanes, Asst. Res. Immunol. I	50%	REDACTED
J. L. Caldwell, Asst. Res. Immunol. I	40%	
C. Henry, Assoc. Res. Immunol. I	25%	
Technical		
D. Aiken, Staff. Res. Associate II	100%	
N. Crise, Staff Res. Associate I	50%	REDACTED
M. Kay, Staff Res. Associate I	25%	
Sub-Total for A		REDACTED
B. Consumable supplies (by major categories)		
Glassware and Chemicals		2,000
Sub-Total for B		2,000
C. Other expenses (itemize)		
Computer programming and multivariant analysis		6,000
Sub-Total for C		6,000
Running Total of A + B + C		53,684
D. Permanent equipment (itemize)		
Sub-Total for D		-0-
E		8,053
E. Indirect costs (15% of A+B+C)		
Total request		61,737

15. Estimated future requirements

	Salaries	Consumable Suppl.	Other. Expenses	Permanent Equip	Indirect Costs	Total
Year 2						
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
ALPHA, ANTI-TRYPSIN AND O OTHER ALPHA-GLOBULIN LEVELS IN SMOKERS AND NONSMOKERS WITH AND WITHOUT EMPHYSEMA	American Medical Association Educational and Research Foundation	$143,687 (total for 3 years)	5-1-71--4/30/74

This grant deals with α anti-trypsin genetic variants, α chymotrypsin levels, and other protease inhibitors in relation to the etiology of emphysema. It does not explore the immunologic approach at all. Except for the use of the same patient population, the AMA-ERF study and the Council for Tobacco Research study do not overlap in any way.

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name H. H. Fudenberg, M.D.

Signature [redacted] Date

Telephone [redacted] (Area Code / Number / Extension)

Checks payable to

Reagents, University of California

Mailing address for checks

Gifts and Endowments
1487 4th Avenue
San Francisco, California 94122

Responsible officer of institution

Typed Name Mrs. Leona M. Butler

Title Contracts & Grants Officer

Signature Leona M. Butler Date

LEONA M. BUTLER

Telephone [redacted]

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